Do developing enteric neurons need endothelins?

Recent experiments have led to the unexpected finding that endothelin-3 and the endothelin B receptor are absolutely necessary for the development of the enteric nervous system in the colon, but it is not yet clear why.

The neural crest has both fascinated and frustrated developmental biologists for a long time [1]. It appears and disappears during embryonic life, giving rise, along the way, to so many different terminally differentiated cell types — including melanocytes, neurons, Schwann cells, and cartilage — in so many distant locations, from the skin to the nervous system, that it has seemed that if one understood the behaviour of the neural crest, one would not be very far from understanding development itself. This accounts for the fascination. The frustration has come from the difficulty scientists have faced in identifying the molecules that serve as signals to crest-derived cells navigating in the embryo and the receptors upon which these molecules act. The frustration has been moderated by a considerable degree of success in learning the routes and destinations of neural crest migration pathways, as well as the knowledge that the behaviour of crest-derived cells is influenced not only by their lineage, but also by the microenvironments in which they differentiate. Recent reports from Masashi Yanagisawa and his colleagues [2–4], demonstrating the critical role played by endothelin-3 and the endothelin B receptor in the development of the enteric nervous system — one of the most complex derivatives of the neural crest — suggest that the frustrating period of molecular ignorance is coming to an end.

The enteric nervous system is different from any other region of the peripheral nervous system. It contains as many neurons as the spinal cord and has more different types of neuron than the ganglia of any other organ [5]. The structure of the enteric nervous system is actually more like that of brain than peripheral nerve; collagen is absent and enteric neurons are supported by glia rather than by Schwann cells. The size and complexity of the enteric nervous system is related to its unique ability to mediate reflex activity in the absence of input from the brain or spinal cord. Microcircuits thus develop in the enteric nervous system that enable it to exert an autonomous control over the secretory and motile behaviour of the bowel.

Only two axial levels of the neural crest, the vagal and the sacral [6], contribute precursors that give rise to the enteric nervous system. Despite this restriction, there is no apparent commitment of crest cells from these levels to differentiate into enteric neurons or glia, nor are they endowed with 'homing' information that enables them to 'find' their correct destinations in the bowel. Instead, these cells migrate along defined pathways and stop in response to signals provided by the enteric microenvironment. The developmental potential of the crest-derived cells is reduced while they migrate, but the cells are still multipotent when they arrive in the bowel [1,7].

The development of the enteric nervous system has been studied extensively in lethal spotted *ls/ls* mice, in an attempt to provide insights into the role played by environmental signals in terminating the migration of crest-derived cells and initiating their differentiation. The most distal portion of the bowel is aganglionic in both *ls/ls* and piebald–lethal (s^l/s^l) mice [8]. The colons of these mice resemble those of human patients with Hirschsprung's disease, in which aganglionosis also occurs. Although the aganglionic segments of murine and human colon contain no nerve cell bodies, they are highly innervated by axons from neurons located outside the gut and in the more proximal bowel [6]. The absence of the intrinsic reflexes of the enteric nervous system from the terminal gut causes the normally innervated bowel proximal to the aganglionic region to dilate, forming a megacolon.

The breakthrough of the Yanagisawa group has been to identify the genetic defects responsible for the development of aganglionosis in both *ls/ls* and s^l/s^l mice, and to show that a related defect is also present in a subset of patients with Hirschsprung's disease. These abnormalities are in genes encoding either the endothelin B receptor [3] or one of its ligands, endothelin-3 [2]. Neither the endothelin B receptor nor endothelin-3 had previously been suspected to be important players in the development of the enteric nervous system. The endothelins (-1, -2 and -3) are a family of 21 amino-acid peptides that activate one or both of the two heptahelical, G-protein-coupled endothelin receptors, A and B [9]. Each of the endothelins has a similar affinity for the endothelin B receptor [10]. Vascular endothelial cells were the first source of endothelins to be discovered and a powerful vasopressor effect was the first endothelin effect to be described [11], but the distribution of the endothelins and their actions are now known to be more extensive [9]. Each endothelin is produced from a large precursor molecule, a preproendothelin, which is first enzymatically processed to an inactive progenitor, called a big endothelin. The big endothelins are then converted to the active peptides by a specific endothelin-converting enzyme [12].

Missense mutations in genes that encode the endothelin B receptor occur spontaneously in s^l/s^l mice (the *ednrb* gene) [3] and in some humans with Hirschsprung's disease (the

EDNRB gene) [4]; moreover, aganglionic megacolon, identical to that seen in s^l/s^l mice, develops in animals with homozygous null mutations in *ednrb* [3]. In *ls/ls* mice, the gene that encodes endothelin-3 (*edn3*) is mutated: an arginine is replaced by a tryptophan residue at the carboxyl terminus of big endothelin-3 [2]. Because of this defect, big endothelin-3 cannot be enzymatically converted to endothelin-3, and big endothelin-3 is inactive. Again, aganglionosis identical to that seen in *ls/ls* mice occurs in animals in which *edn3* is 'knocked out' with a targeted mutation [2]. The landmark studies of the Yanagisawa group thus make absolutely clear that both the ligand, endothelin-3, and the endothelin B receptor, are necessary for the development of the enteric nervous system in the terminal bowel. The experiments leave unsolved, however, exactly how the interaction of endothelin-3 with endothelin B receptor affects the development of enteric neurons and why the absence of this interaction affects enteric neurogenesis only in the colon. One of the most important pieces of the puzzle that is still not in place is the identification, in the fetal colon at the critical time in development, of the cells that express endothelin-3 and the endothelin B receptor.

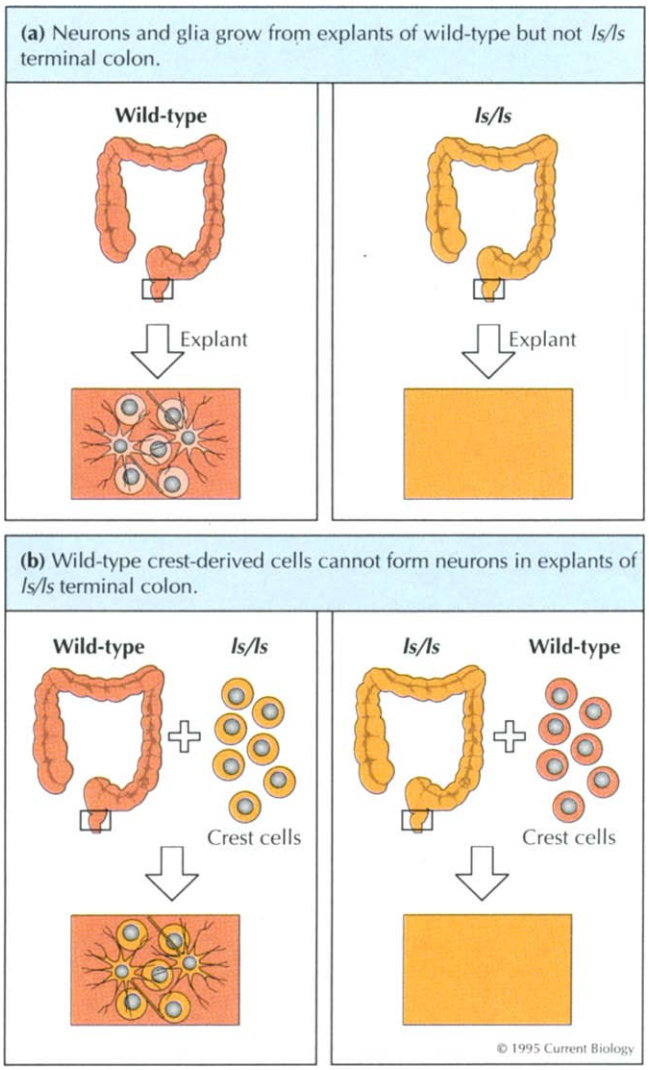

Fig. 1. Co-culture experiments suggest that the defect responsible for aganglionosis in *ls/ls* mice is microenvironmental.

As each of the endothelins has approximately the same affinity for the endothelin B receptor [10], one would assume that endothelin-1 or endothelin-2 would compensate for the specific deficiency of endothelin-3 in *ls/ls* or $edn3^{-/-}$ mice, as long as these ligands were able to reach the relevant endothelin B receptors. That they do not do so suggests that the developmental action of endothelin-3 is a local one, and that the endothelins do not circulate in the fetus [2]. Yanagisawa and co-workers [2] note that, in addition to their aganglionosis, *ls/ls*, s^l/s^l, $edn3^{-/-}$ and $ednrb^{-/-}$ mice all have white spots, in which melanocytes fail to develop, and they propose that endothelin-3 is an autocrine growth factor essential for the development of crest-derived cells such as enteric neurons and melanocytes. This hypothesis is plausible and, in the absence of evidence as to which cells express endothelin-3 or endothelin B receptors, must be seriously considered; however, the hypothesis does not explain why aganglionosis occurs only in the colon, nor does it account for the results of previously published experiments that have suggested that the lesion in *ls/ls* mice is not crest-autonomous [13–15].

As enteric neurons develop normally in the esophagus, stomach, and small intestine of animals that lack either endothelin-3 or endothelin B receptors, neither factor would seem to be essential for the manifestation of the enteric neuronal lineage *per se*. Potential local targets for the action of endothelin-3 in the colon, however, are not limited to incoming crest-derived cells. As crest-derived cells are known to be sensitive to the microenvironment they find in the bowel, almost any cell that contributes to this microenvironment could be endothelin-3-dependent and could thus transmit the effects of an endothelin-3 deficiency to the crest-derived cells. For example, intestinal smooth muscle in adult animals is known to express endothelin B receptors and to be a target of endothelin-3 [16]; it is therefore possible that developing smooth muscle, or smooth muscle precursors, could be a target of endothelin-3 in the fetal colon, and that smooth muscle cells might, in turn, influence the development of the enteric nervous system. The endothelin-3 deficiency in *ls/ls* mice could thus cause incoming crest-derived cells to fail to complete their colonization of the bowel, not by acting directly on these cells but by affecting the development of one of the cells of the enteric mesenchyme.

The suggestion that a microenvironmental defect might be responsible for aganglionosis of the *ls/ls* colon first arose from our studies of enteric neuronal development in cultured explants of bowel [17]. Although neurons and glia develop in such cultures of explants taken from control gut, neither neurons nor glia ever develop in cultures of the terminal 2 mm of *ls/ls* bowel (Fig. 1a). Crest-derived cells from almost any source, moreover, including cells from the foregut of *ls/ls* mice, form neurons in explants of colon from control mice, but no crest-derived

cells, from any source, form neurons in explants of *ls/ls* colon (Fig. 1b) [18]. More recently, the colon has been found to be normally ganglionated in aggregation chimeras constructed between *ls/ls* and control mice [14,15]; furthermore, *ls/ls* neurons, identified in such chimeric fetuses using either an endogenous [15] or a transgenic [14] marker, were observed in even the most distal portion of the colon. Indeed, the progression of transgenically-labeled vagal crest-derived cells down the *ls/ls* bowel is quite normal until they enter the colon [13,19].

None of these earlier experiments can be straightforwardly explained by the hypothesis that endothelin-3 is an autocrine factor that is necessary for the development of crest-derived cells into enteric neurons. For example, it is not clear why endothelin-3-deficient *ls/ls* crest-derived cells, which should be unable to provide themselves with the proposed autocrine stimulation, are able to colonize a wild-type colon. Nor is it clear why wild-type crest-derived cells, which would be expected to stimulate themselves normally, are unable to colonize an *ls/ls* colon. Similar difficulties are encountered in accounting for the results of back-transplantation experiments in which segments of wild-type or *ls/ls* colon were grafted into neural crest migration pathways in quail host embryos [15]. Quail crest-derived cells enter and migrate through such grafts from wild-type mice; but the quail crest-derived cells fail to enter the *ls/ls* tissue. Experimental results are therefore consistent with the possibility that the crest-derived cells of *ls/ls* mice are capable of forming enteric neurons in the colon as well as in the proximal bowel, but that the absence of endothelin-3 causes the colon to become refractory to colonization, even by normal crest-derived cells.

The idea that the endothelin-3 deficiency of *ls/ls* mice leads to defects in the wall of the colon is supported by observations that have indicated that the extracellular matrix (ECM) is defective in these animals. An overabundance and/or maldistribution, of laminin, type IV collagen, non-sulfated glycosaminoglycans, and proteoglycans have all been found [20,21]. The abnormal *ls/ls* ECM is located in what would be predicted to be the pathways of both vagal crest-derived cells migrating within the bowel and sacral crest-derived cells approaching the gut. The overabundance of ECM molecules seems to be present before crest-derived cells normally enter the developing colon, and the lesion does not occur in mice that have a targeted mutation in the proto-oncogene c-*ret* (T. Rothman, J. Chen, M. Howard, F. Costantini and M.G., unpublished observations), in which the bowel is aganglionic for a reason quite separate from the *ls/ls* defect [22]. The same ECM abnormalities as have been reported in *ls/ls* mice have also been observed in human patients with Hirschsprung's disease [23]. These observations all suggest that the ECM defects in the *ls/ls* (and Hirschsprung's disease) colon, like the aganglionosis, are caused by the failure of endothelin-3 to stimulate endothelin B receptors expressed by one of the cells of the colonic mesenchyme.

It is possible that one or more of the abnormalities of the ECM in *ls/ls* mice could be causally related to the development of aganglionosis. Laminin, for example, promotes the development of enteric neurons and glia [6]. When overexpressed, laminin might induce crest-derived cells to differentiate prematurely, thereby stopping their migration before they have finished colonizing the bowel. Whether or not this is the case, the incidence of ECM defects in the *ls/ls* and Hirschsprung's disease colon certainly indicates that cells of neural crest origin are not the only ones to suffer developmental consequences from the failure of endothelin-3 to stimulate endothelin B receptors.

In summary, the focus of research on the development of the enteric nervous system, and indeed of every cell of neural crest origin, has been permanently changed by the studies of the Yanagisawa group [2–4]. The endothelins and their receptors have made their debut, and now they will appear on the agenda of virtually every scientist in the field. The Yanagisawa papers are absolutely clear and remarkably complete; nevertheless, they raise many new questions about developmental mechanisms that cry out for answers. Although no observations, past or present, are contradictory, the data can be interpreted in different ways. Yanagisawa and his co-workers have thus provided their colleagues with an intriguing new set of issues to address.

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Michael D. Gershon, Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032, USA.